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Interdependency of MHC Class II/Self-Peptide and CD1d/Self-Glycolipid Presentation by TNF-Matured Dendritic Cells for Protection from Autoimmunity

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Dendritic cells (DCs) are key regulators of T cell immunity and tolerance. NKT cells are well-known enhancers of Th differentiation and regulatory T cell function. However, the nature of the DC directing T and NKT cell activation and polarization as well as the role of the respective CD1d Ags presented is still unclear. In this study, we show that peptide-specific CD4+IL-10+ T cell-mediated full experimental autoimmune encephalomyelitis (EAE) protection by TNF-treated semimatured DCs was dependent on NKT cells recognizing an endogenous CD1d ligand. NKT cell activation by TNF-matured DCs induced high serum levels of IL-4 and IL-13 which are absent in NKT cell-deficient mice, whereas LPS plus anti-CD40-treated fully mature DCs induce serum IFN-γ. In the absence of IL-4Rα chain signaling or NKT cells, no complete EAE protection was achieved by TNF-DCs, whereas transfer of NKT cells into Jb281+/- mice restored it. However, activation of NKT cells alone was not sufficient for EAE protection and early serum Th2 deviation. Simultaneous activation of NKT cells and CD4+ T cells by the same DC was required for EAE protection. Blocking experiments demonstrated that NKT cells recognize an endogenous glycolipid presented on CD1d on the injected DC. Together, this indicates that concomitant and interdependent presentation of MHC II/self-peptide and CD1d/self-isoglobotrihexosylceramide to T and NKT cells by the same partially or fully matured DC determines protective and nonprotective immune responses in EAE. The Journal of Immunology, 2007, 178: 4908–4916.

Recent work has provided evidence for the importance of dendritic cells (DCs) for peripheral tolerance and thus for immunotherapy of autoimmune diseases (1, 2). Regulatory T cells (Tregs) contribute in the establishment of peripheral tolerance (3). In contrast to thymus-derived CD4+CD25+ Tregs, regulatory type 1 T (Tr1) cells are induced from naive cells in the periphery and regulate mainly through IL-10. A role for DCs in peripheral tolerance induction by Tr1 cells has been proposed in several recent studies (4, 5). However, the precise mechanisms regarding how Tr1 cells develop and function in vivo remain elusive.

The majority of NKT cells, which express a TCR and the NK cell marker NK1.1, are characterized by a highly homologous invariant TCR (6–8). In mice as well as in humans, these invariant TCR molecules recognize glycolipids in a CD1d-restricted manner (9). Upon activation, NKT cells rapidly secrete large amounts of cytokines such as IFN-γ, IL-4, IL-10, and IL-13 (10), thereby influencing the adaptive immune response toward a Th1 or Th2 profile (11). However, several experimental in vivo models using NKT cell-depleted or NKT-deficient mice showed an unaltered Th1/Th2 balance, suggesting that they are not prerequisite for Th1/Th2 induction (12, 13). The observation that patients with the autoimmune disease multiple sclerosis have a reduced frequency and function of NKT cells may indicate a role of these cells in T cell tolerance (14, 15). The ability of CD1d-restricted cells to inhibit self-reactive immune responses has been demonstrated in animal models of organ-specific autoimmune diseases such as type I diabetes and experimental autoimmune encephalomyelitis (EAE) (16–23). However, their precise mechanism remains often elusive and conditions modulating NKT cell function are controversial in the literature, describing induction of Th1 or Th2 responses as well as nonresponsiveness (19, 20, 24–27). The outcome of NKT cell activation depends also on the interaction with APCs by costimulatory molecules like CD40/CD40L (28, 29).

To date only limited endogenous CD1d ligands are known, however, Jahng et al. (30) described a myelin-derived glycolipid, sulfatide, which is recognized by a distinct subset of CD1d-reactive cells able to prevent EAE. Recently, Zhou et al. (31) identified the lysosomal glycosphingolipid isoglobotrihexosylceramide (iGb3) as a potential endogenous CD1d ligand recognized by human as well as mouse NKT cells. So far, α-galactosylceramide (α-GalCer) isolated from a marine sponge is generally used as an exogenous surrogate Ag to activate NKT cells (32). Thus, little is known...
about the exact DC types and the role of endogenous CD1d ligands directing NKT cell function in T cell tolerance.

Previously, we have reported that bone marrow (BM)-derived DCs treated with TNF-α (TNF-DCs) achieve only a partially matured phenotype (33). Although these TNF-DCs up-regulate MHC class I (MHC I)/II, CD80, CD86, and CD40, they did not secrete cytokines in contrast to fully matured DCs stimulated with LPS plus anti-CD40 (LPS plus CD40-DCs). Repetitive injections of TNF-DCs suppress EAE in C57BL/6 mice in an Ag-specific manner mediated by IL-10+ CD4+ T cells (33). Interestingly, such TNF-DCs prevent also autoimmune diseases like thyroiditis and rheumatoid arthritis, conforming their tolerogenic capacity (34, 35).

In this study, we found that an Ag-specific protective CD4+ T cell response induced by TNF-DCs was dependent on CD1d-resticted NKT cells without usage of an exogenous CD1d ligand. Simultaneous activation of Ag-specific CD4+ T and innate NKT cell responses by MHC II/self-peptide and CD1d/self-glycolipid, respectively, by the same DC interdependently controls induction of protective and nonprotective immune responses.

Materials and Methods

Mice

All mice were bred and housed at the Department of Dermatology, University of Erlangen, C57BL/6 (Charles River Laboratories), CD1d−/−(provided by L. van Kaer, Vanderbilt University School of Medicine, Nashville, TN), Jc281+/+ (provided by M. Taniguchi, Institute of Physical and Chemical Research, Kanagawa, Japan), and C57BL/6tg mice (H11002) were backcrossed on a C57BL/6 genetic background for more than eight generations. All animal experiments were performed in accordance with institutional guidelines with age- and sex-matched animals.

Generation and maturation of BM-DCs

DCs were generated from BM cells derived from C57BL/6, MHC II−/−, or CD1d−/− mice and matured with TNF-α (500 U/ml; PeproTech) or with LPS (1 μg/ml; Sigma-Aldrich; Escherichia coli, 027;BB) and anti-CD40 (clone PGK-45; hybridoma supernatant) together with 10 μM myelin oligodendrocyte glycoprotein (MOG)35–55 peptide (Sigma-Genosys) as described previously (33). To block IgG3 recognition by NKT cells, DCs were differentially matured with or without 10 μg/ml Griffonia simplicifolia isoelectin B4 (B4; Vector Laboratories).

Induction of EAE and injections of DCs

EAE induction and DC injections were conducted essentially as described (33). Briefly, mice were injected i.c. with MOG35–55 peptide (100 μg/mouse) emulsified in CFA (Sigma-Aldrich) that was further enriched with Mycobacterium tuberculosis (H37Rv; Difco/BD Pharmingen). In addition, pertussis toxin (List/Quadrature) was injected i.p. at days 0 and 2. Mice were observed daily for clinical signs of disease and scored according to their clinical severity of disease as follows: grade 0, no abnormality; grade 1, limp tail or hind limb weakness; grade 2, limp tail and hind limb weakness; grade 3, partial hind limb paralysis; grade 4, complete hind limb paralysis; and grade 5, moribund. The data are plotted as the mean daily clinical score for all animals per group. A total of 2–2.5 × 106 DCs were injected i.v. at days −7, −5, and −3 before EAE induction. When the mixture of MHC II−/− DCs and CD1d−/− DCs was used, 2.5 × 106 DCs of each DC type were injected.

Measurement of proliferative and cytokine response

For proliferative and cytokine responses, 4 × 106 splenocytes depleted of erythrocytes were cultured in vitro in the presence of graded concentrations of MOG35–55 peptide (0–20 μM). Supernatants were collected after 72 h and cytokines produced from spleen cells were detected using ELISA kits for IL-4, IL-10, IFN-γ (all from BD Pharmingen), and IL-13 (R&D Systems). Proliferation was assayed by [3H]thymidine incorporation for an additional 18 h. Sera collected at the indicated time points or 2 h after the third immunization were analyzed for cytokine production by using ELISA kits.

Adoptive transfer of NKT cells

Erythrocyte-depleted spleen cells from Va14xJ281 tg mice were labeled with anti-CD8 (53–6.7, -5, and -2B20) followed by magnetic depletion with anti-rat Ig coated with Dynabeads (Dynal Biotech). For flow cytometric sorting, enriched cells were surface labeled with anti-αβTCR-FITC (H57–597) and α-GalCer-loaded CD1d-Pe tetrabromomethane (pro-duced as described by others (37)). All Abs were purchased from BD Pharmingen unless otherwise indicated. After cell surface labeling, cells were sorted using MoFlo (DakoCytomation). Purity was always assessed and resulted in αβTCR+CD1d tetramer+ (NKT cell) populations of ≥96%. One day before first DC injection, 1.5–2 × 106 sorted NKT cells were injected i.v. into each Jc281+/+ mouse.

Flow cytometric analysis

To avoid unspecified binding of Abs to FcγR, cells preincubated with 2.4G2 (CD16/32; hybridoma supernatant) or with medium containing 10% serum before cells were stained with the following mAbs: anti-CD3-PerCP (145-2C11), anti-CD4-FITC (GK1.5), anti-NK1.1-PE (PK136), anti-MHC II-PE (M5/114), and anti-CD1d-IFC (B11). All Abs were purchased from BD Pharmingen.

Detection of intracellular cytokines by flow cytometric analysis

Splenocytes from mice immunized twice with DC were depleted of erythrocytes followed by B cell depletion using B220 Ab conjugated to magnetic beads (Dynal) to enrich T and NKT cells. Residual cells were suspended at 4 × 106/ml and stained with MOG/TNF-DC (4 × 106/ml) for 5 h. Brefeldin A (Sigma-Aldrich) was added for the final 3 h. Then, cells were washed in PBS/2% FCS and stained with anti-CD4-Fluorescein isothiocyanate (FITC), anti-CD1d-PerCP, anti-NK1.1-PerCP-Cy5.5 (PK136; all from BD Pharmingen), and α-GalCer-loaded CD1d-allophyocycocyanin tetrabromomethane (α-GalCer was provided by Kirin Brewery). Unspecific binding was blocked by preincubating anti-FcγRII/III (2.4G2, hybridoma supernatant). For intracellular cytokine detection, cells were fixed with 2% formaldehyde, permeabilized with Perm/Wash (BD Pharmingen), and stained with the following mAbs conjugated to PE: anti-IL-4 (11B11), anti-IFN-γ (XMGL-21), anti-IL-10 (JES5-16E3; all from BD Pharmingen), and anti-IL-13 (38213.11; R&D Systems). Isotype control mAbs (BD Pharmingen) were used at the same concentration.

Statistical analysis

Statistical significance of difference in cytokine secretion between groups was analyzed using Student’s t test and significance was accepted if p < 0.05. Data of EAE experiments were statistically validated by Wilcoxon test and Fisher’s exact test.

Results

EAE tolerance induced by TNF-DCs depends on their expression of CD1d and activation of NKT cells

We have demonstrated previously that repeated injections of DCs stimulated with TNF-α and loaded with MOG peptide (MOG/ TNF-DCs) prevent EAE, partially by induction of IL-10+ CD4+ T cells (33). To further analyze cellular requirements for this tolerance, we focused on NKT cells based on our findings that the splenic NKT cell population was slightly expanded following treatment with MOG/TNF-DCs (data not shown). This finding was surprising because none of the injected DC types was loaded with an exogenous CD1d Ag and both expressed comparable levels of CD1d on their surface (data not shown). Although the frequency of NKT cells increased only modestly, their role was further investigated by applying the EAE tolerance protocol to CD1d−/− mice deficient for NKT cells (38). As reported earlier (33), three i.v. injections of MOG/TNF-DCs protected C57BL/6 mice from EAE in an Ag-specific manner and only 2 of 50 mice showed signs of EAE (Fig. 1A, left panel, and Table I). In contrast, 47 of 49 control mice and 42 of 46 mice injected with MOG-pulsed LPS plus CD40-DCs (MOG/LPS plus CD40-DCs) developed severe EAE. However, MOG/TNF-DCs generated from WT mice were unable to prevent EAE in 8 of 8 CD1d−/− mice lacking NKT cells (Fig. 1A, right panel, and Table I). Furthermore, injection of wild-type (WT) MOG/TNF-DCs did not also suppress EAE when injected into Jc281+/+ mice (10 of 13 mice developed EAE, Table I), suggesting that the expression of CD1d was not sufficient to suppress EAE.
FIGURE 1. CD1d-restricted NKT cells and CD1d expression on the injected MOG-pulsed TNF-DCs control prevention of EAE. A. Before EAE induction (day 0), C57BL/6 or CD1d−/− mice were injected three times i.v. with MOG-pulsed TNF-DCs or LPS+CD40-DCs (days −7, −5, −3). Control mice were injected with PBS and paralysis was monitored. B. EAE prevention in Jα281−/− mice induced by MOG/TNF-DCs was reconstituted by transfer of NKT cells. Before EAE induction (day 0), Jα281−/− mice were reconstituted with NKT cells (day −8) and then repetitively injected with MOG/TNF-DCs. Three days after the last injection, EAE was induced and paralysis analyzed. C. CD1d−/− mice were injected three times i.v. with MOG/TNF-DCs generated either from C57BL/6 (WT) or CD1d−/− mice. Following EAE induction (day 0), clinical symptoms were analyzed and compared with control mice (PBS). Results are represented as average of four mice per group, representative of at least two independent experiments.

lacking the population of Vα14-Jα18 (formerly Jα281) NKT cells (Fig. 1B). To further verify the need for NKT cells, Jα281−/− mice were reconstituted with CD1d-restricted NKT cells before DC injections and subsequent EAE induction. In contrast to Jα281−/− mice injected with MOG/TNF-DCs, which developed EAE, adoptive transfer of NKT cells restores the tolerogenic capacity of TNF-DCs resulting in EAE prevention and only 1 of 6 mice developed EAE (Fig. 1B and Table I). In addition, the reverse experimental setting, where MOG/TNF-DCs derived from CD1d−/− mice were used to treat C57BL/6 mice, proved that CD1d deficiency of the injected TNF-DCs is sufficient to significantly revert complete EAE protection induced by WT DCs and 8 of 13 animals showed signs of EAE (Fig. 1C and Table I).

Together, this indicates that in the EAE model, where MOG55–55 peptide-specific IL-10+CD4+ T cells act as tolerogenic effectors, the presence of CD1d-restricted NKT cells is required to optimize EAE prevention induced by TNF-DCs in the absence of an exogenous ligand for CD1d. Thus, NKT cell activation by the injected TNF-DCs is crucial for their function to induce EAE tolerance.

**Activation of NKT via CD1d on TNF-DCs creates a type 2 cytokine environment in the serum**

The predominant immune function of NKT cells is their rapid secretion of large amounts of cytokines, which can be measured in the serum and are considered to modulate immune responses by shifting the balance between Th1 and Th2 cells (11). Using α-GalCer, a surrogate ligand for NKT cells, serum IFN-γ and IL-4 were detectable already 2 h after the first i.v. injection whereas MOG-pulsed DCs had to be injected three times (data not shown). C57BL/6 mice treated with fully mature MOG/LPS plus CD40-DCs displayed a Th1-like serum cytokine profile with high IFN-γ and low levels of IL-4 and IL-13 measured 2 h after the third injection (Fig. 2A). In contrast, MOG/TNF-DCs induced a Th2-like cytokine profile characterized by high levels of IL-4 and IL-13 and low to intermediate IFN-γ (Fig. 2A). IL-10 was not detectable in the sera of mice after injection of either DC type (Fig. 2A and data not shown). These cytokine profiles suggest that protective TNF-DCs induce a Th2 cytokine environment whereas nonprotective LPS plus CD40-DCs rather mediate a Th1 cytokine environment.

IL-4 and IL-13 serum levels were undetectable when CD1d-deficient MOG/TNF-DCs were injected into C57BL/6 while serum levels for IFN-γ remained unaffected (Fig. 2B). This suggests that CD1d-restricted cells are the source of IL-4 and IL-13. To analyze whether the invariant NKT cell subset contributes to the CD1d-dependent IL-4 and IL-13 secretion, Jα281−/− mice were treated with MOG/TNF-DCs. In contrast to C57BL/6 mice, serum IL-4 and IL-13 were significantly diminished in Jα281−/− mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence</th>
<th>Mean Maximal Score</th>
<th>Mean Day of Onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 (WT)</td>
<td>47/49 (96%)</td>
<td>3.1 ± 1.2</td>
<td>12.8 ± 2.4</td>
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<td>MOG/TNF-DC into WT</td>
<td>2/50 (4%)</td>
<td>0.0 ± 0.2</td>
<td>13.5 ± 0.7</td>
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<tr>
<td>MOG/LPS plus CD40-DC into WT</td>
<td>42/46 (91%)</td>
<td>2.5 ± 1.3</td>
<td>9.9 ± 2.2</td>
</tr>
<tr>
<td>MOG/TNF-DC into CD1d−/−</td>
<td>8/8 (100%)</td>
<td>3.7 ± 1.6</td>
<td>12.5 ± 0.9</td>
</tr>
<tr>
<td>MOG/TNF-DC into Jα281−/−</td>
<td>10/13 (77%)</td>
<td>2.1 ± 1.7</td>
<td>17.4 ± 4.1</td>
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<tr>
<td>MOG/TNF-DC plus iNKT cells into Jα281−/−</td>
<td>1/6 (17%)</td>
<td>0.2 ± 0.4</td>
<td>13.0 ± 2.4</td>
</tr>
<tr>
<td>MOG/TNF-DC into IL-4α−/−</td>
<td>6/8 (75%)</td>
<td>1.4 ± 1.1</td>
<td>16.5 ± 3.7</td>
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<tr>
<td>MOG/TNF-DC (CD1d−/−) into WT</td>
<td>8/13 (61%)</td>
<td>1.7 ± 1.6</td>
<td>14.6 ± 1.6</td>
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<tr>
<td>MOG/TNF-DC (MHC II−/−) into WT</td>
<td>13/15 (87%)</td>
<td>2.7 ± 1.3</td>
<td>13.9 ± 1.9</td>
</tr>
<tr>
<td>Mix of MOG/TNF-DC (CD1d−/−) plus MOG/ TNF-DC (MHC II−/−) into WT</td>
<td>6/10 (60%)</td>
<td>1.6 ± 1.7</td>
<td>14.8 ± 3.5</td>
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</table>

* Mice were injected i.v. (days −7, −5, −3) with MOG/TNF-DC, MOG/LPS-DC, or left untreated. On day 0 mice were immunized s.c. with MOG/CFA plus pertussis toxin (i.p. on days 0 and 2). The incidence, mean maximal score (±SE), and mean day of onset of clinical EAE (±SE) is shown. Statistically significant when compared with C57BL/6; p < 0.0001 by Wilcoxon rank-sum test for mean clinical score and p < 0.0001 by Fisher’s exact test for incidence.

* Statistically significant when compared with MOG/TNF-DC in WT; p < 0.01 by Wilcoxon rank sum test for mean clinical score and p < 0.001 by Fisher’s exact test for incidence.

* Statistically significant when compared with MOG/TNF-DC in WT; p < 0.01 by Wilcoxon rank-sum test for mean clinical score and p < 0.001 by Fisher’s exact test for incidence.
C57BL/6 mice treated with WT MOG/TNF-DCs were compared with naive C57BL/6 mice. Three independent experiments demonstrated that CD1d-expressed on injected TNF-DCs activate invariant NKT cells resulting in secretion of Th2 cytokines IL-4 and IL-13 while LPS plus CD40-DCs reversely induce the Th1 cytokine IFN-γ.

**NKT cells produce Th2 cytokines and enhance Th2 cytokine secretion by CD4+ T cells**

To identify the cell type responsible for the serum cytokines following DC injections, B cell-depleted splenocytes from mice injected twice with DCs were stimulated in vitro with MOG/TNF-DCs. Then, cytokine production was analyzed by intracellular cytokine staining. In contrast to Jo281+ T cells, splenocytes from C57BL/6 mice contained NKT cells, identified as CD1d tetramer-positive cells and these tetramer+ cells were also positive for NK1.1 (Fig. 3B). When splenocytes from naive C57BL/6 were cocultured with MOG/TNF-DCs, no cytokine-producing cells could be detected (Fig. 3A), indicating that in vitro stimulation was not sufficient to induce cytokine production. Splenocytes from C57BL/6 injected twice with DCs contained a small proportion of CD1d tetramer+ NK1.1+ cells producing IFN-γ, IL-4, and IL-13 (Fig. 3B, middle column), which were detectable in the sera after the third DC injection (Fig. 2A). However, there was also substantial production of these cytokines by CD4+ T cells (Fig. 3B, left column), suggesting that NKT cells are not the only source of these cytokines. Interestingly, the frequencies of IL-4- and IL-13-producing CD4+ T cells were reduced in spleen cells from Jo281+ mice while IFN-γ CD4+ T cells remained unaffected (Fig. 3B, right column). This finding is in line with the observed reduction of Th2 cytokines in the sera of mice injected with MOG/TNF-DCs without NKT cell activation (Fig. 2, B and C), and supporting that “classical” CD4+ T cells might be the major source of the serum cytokines.

These findings show that MOG/TNF-DCs activate CD1d-restricted NKT cells to secrete cytokines like IL-4, IL-13, and IFN-γ. Moreover, CD4+ T cells are a substantial source of these cytokines and, interestingly, their efficient IL-4 and IL-13 production depend on simultaneous NKT cell activation, suggesting a critical regulatory role of NKT cells in Th2 differentiation of CD4+ T cells.

**EAE suppression and induction of CD4+ IL-10+ T cells rely on IL-4Rα chain signaling**

We wondered whether the CD1d-dependent serum Th2 cytokines IL-4 and IL-13 functionally contribute to MOG-specific EAE prevention induced by TNF-DCs. Therefore, IL-4Rα−/− mice, which are unresponsive to IL-4 and IL-13 (39), were injected with MOG/TNF-DCs or MOG/LPS plus CD40-DCs generated from WT mice before induction of EAE. Injection of MOG/TNF-DCs into IL-4Rα−/− mice did not lead to complete EAE protection, whereas IL-10−/− mice. Moreover, also CD4+ T cells are a substantial source of these cytokines and, interestingly, their efficient IL-4 and IL-13 production depend on simultaneous NKT cell activation, suggesting a critical regulatory role of NKT cells in Th2 differentiation of CD4+ T cells.

Together, the early CD1d-dependent Th2 cytokine environment created by NKT cells and “classical” CD4+ T cells is functionally important for EAE suppression and development of MOG-specific IL-10−/− CD4+ T cells. Although we detected NKT cells, which produce the cytokines detectable in the serum, the questions remained regarding whether NKT cells or CD4+ T cells are the major source of the serum cytokines, and also as to how Ag specificity is achieved in this tolerance model mediated by MOG-specific CD4+ T cells (33).

**CD1d-restricted NKT cells alone are unable to protect from EAE following TNF-DC injections**

To investigate whether NKT cells alone are sufficient for EAE prevention by directly modifying the MOG-specific T cell response, we injected MHC II-deficient MOG/TNF-DCs into WT mice to abolish any interaction of the applied DC with CD4+ T cells at the time point of NKT cell activation. Similar to the results obtained with CD1d−/−DCs (Fig. 1C), even 13 of 15 C57BL/6 mice treated with MHC II−/− MOG/TNF-DCs developed severe EAE.
like control mice (Fig. 5A and Table I). In addition, these mice lacked serum IL-4 and IL-13 (Fig. 5B) as observed in sera of mice without NKT cell activation (Fig. 2, B and C). However, in contrast to these cytokine profiles, serum IFN-γ levels were also reduced with MHC II−/−-DCs (Fig. 5B). As a further consequence, the T cell cytokine profile of spleen cells after MOG peptide re-stimulation shifted from the protective IFN-γlowIL-10high pattern to the nonprotective IFN-γhighIL-10low phenotype (Fig. 5, C and D). To rule out that these results were not caused by the failure of MHC II−/−-DCs to activate NKT cells, the ability of these DCs to activate NKT cells after injections was confirmed by CD1d tetramer and cytokine staining (data not shown).

**FIGURE 3.** Splenic NKT cells and CD4+ T cells both contribute to the early IL-4 and IL-13 production. Splenocytes were isolated (day 0) from naïve C57BL/6 (A) or C57BL/6 and Jo281−/− mice which were injected twice (days −4, −2) i.v. with MOG/TNF-DCs (B). Following depletion of B cells, residual cells were cocultured in vitro for 5 h with MOG/TNF-DCs in the presence of brefeldin A for the last 3 h. Cells were stained for indicated surface Ags and intracellular cytokines. The inserted numbers represent percentages of α-GalCer-CD1d tetramers+ or CD4+ cells, respectively, generating a given cytokine. The results shown are representative of two independent experiments.

**FIGURE 4.** EAE prevention and induction of MOG-specific CD4+ IL-10+ T cells by TNF-DCs depend on IL-4Rα chain signaling. C57BL/6 or IL-4Rα−/− mice were injected three times (days −7, −5, −3) i.v. with MOG-pulsed TNF-DCs or LPS+CD40-DCs. Control mice were left untreated. A, Following EAE induction (day 0), clinical symptoms were monitored. On day 31 after EAE induction, isolated spleen cells were restimulated in vitro with graded concentrations of MOG peptide for 72 h. B, Proliferation was assayed by [3H]thymidine incorporation for an additional 18 h. Harvested cell supernatants were analyzed for MOG-specific secretion of IFN-γ (C) and IL-10 (D) by ELISA. Results are represented as the average of four mice combined, representative of at least two independent experiments.

**FIGURE 5.** EAE prevention relies on both CD1d- and MHC II-restricted pathways. C57BL/6 mice were repetitively injected (days −7, −5, −3) i.v. with MOG/TNF-DCs either generated from C57BL/6 (WT) or MHC II−/− mice. Control mice were left untreated. A, Clinical EAE symptoms were analyzed after disease induction on day 0. B, Two hours post-third DC injection, sera were collected and cytokines were measured by ELISA. *, Significant differences (p < 0.05) from mice treated with WT MOG/TNF-DCs. On day 31 after EAE induction, spleen cells were in vitro restimulated with various concentrations of MOG. Harvested cell supernatants were analyzed for MOG-specific secretion of IFN-γ (C) and IL-10 (D) by ELISA. Results are representative for at least three independent experiments with four mice per experimental condition.
Thus, NKT cell activation alone by MOG/TNF-DCs is not sufficient to suppress EAE, thereby restricting the Ag specificity to MHC II/peptide presented on DCs. The complete reduction of all serum cytokines induced by MHC II $^-/-$-DCs identifies MHC II-restricted CD4$^+$ T cells as a major source of these cytokines, which is supported by the higher number of cytokine$^+$CD4$^+$ T cells compared with NKT cells (Fig. 3B).

**Regulatory effect of NKT cells relies on injected TNF-DCs expressing both MHC II and CD1d**

To prove whether the regulatory effect of NKT cells requires feedback signaling toward the injected TNF-DCs, mice were treated with a mixture of DCs containing the normal number (2.5 $\times$ 10$^5$ mouse) of MHC II$^+$CD86$^+$-DCs plus the same number CD1d$^+$-DCs. Six of 10 mice injected with the DC mixture developed EAE to a similar degree as animals treated with CD1d-deficient MOG/ TNF-DC only, but not as severe as those treated with MHCII$^-/-$-DCs (Fig. 6A and Table I). These mice also showed a serum cytokine profile identical with that one with CD1d$^+$-DCs, displaying significantly reduced levels of IL-4 and IL-13 but similar IFN-$\gamma$ levels compared with WT-DC (Fig. 6B). Subsequently, we investigated the capacity of splenic T cells to produce IL-10 following MOG-specific in vitro restimulation 30 days post-EAE induction. Only spleen cells from mice treated with WT MOG/ TNF-DCs exhibited increased IL-10 secretion, whereas treatment with CD1d$^-/-$ or MHC II$^-/-$-DCs alone as well as mixed together resulted in reduced IL-10 levels (Fig. 6C).

These data indicate that both MHC II and CD1d have to be expressed on the same DC for the early Th2-biased cytokine environment followed by EAE suppression and development of MOG-specific IL-10$^+$ T cells. Thus, rather than a direct effect on the CD4$^+$ T cells, it suggests that the NKT cell regulatory effect might rely on feedback signaling toward DCs, a mechanism described for CD4$^+$ T cells as “DC education” for tolerogenic CD4$^+$ responses (40) or “licensing” for CD8$^+$ immunity (41).

**Activation of CD1d-restricted NKT cells by injected DCs depends on presentation of endogenous CD1d ligand**

Finally, we addressed the question about the CD1d ligand presented by the injected DCs, because no exogenous ligand such as $\alpha$-GalCer or even EAE-related sulfatides were used (30). Recently, Zhou et al. (31) identified iGb3 as endogenous CD1d ligand recognized by human and mouse NKT cells. IB4, which specifically binds to the terminal Gal1,3Gal linkage, blocked CD1d presentation of exogenous and endogenous iGb3 (31, 42). Therefore, the impact of IB4 blockade experiments on the early cytokine environment and EAE suppression was studied. IL-4 and IL-13 serum levels were significantly decreased when IB4-treated MOG/TNF-DCs were injected into C57BL/6 mice while levels for IFN-$\gamma$ remained uninfluenced (Fig. 7A), a cytokine profile similar as in the absence of NKT activation (Fig. 2, B and C). This is in contrast to MOG/ LPS plus CD40-DCs. Here, IB4 resulted in significantly reduced levels of serum IFN-$\gamma$ whereas IL-4 and IL-13 were unaffected. To further demonstrate the CD1d dependency of this IB4 effect on serum cytokines, WT and CD1d$^-/-$ DCs were compared. When CD1d$^-/-$ DCs were pretreated with IB4, no additional reduction of IL-4 and IL-13 was measured compared with IB4-treated WT TNF-DCs (Fig. 7B). In addition, the IB4 effect was also not due to altered maturation of DCs, because surface marker expression and...
cytokine secretion were unaffected by IB4 (Fig. 7, C and D). The treatment with IB4 showed no effect on EAE (data not shown), which might be due to the short half-life and/or low-affinity interaction being unable to block NKT cell activation over a longer period.

Overall, these data demonstrate that an endogenous CD1d ligand with terminal Galα1,3Gal, such as iGb3, is presented on both types of differentially matured DC and may result in NKT cell activation. This CD1d/self-glycolipid pathway is of crucial regulatory function for the concomitant MHC II-dependent T cell activation on the same DC by 16 enhancing the early transient Th2 differentiation, which counteracts the Th1-mediated autoimmunity of EAE and promotes development of protective MOG-specific IL-10+CD4+ T cells.

**Discussion**

Recently, we and others have described that DCs sense inflammatory and microbial signals differently and while both signals result in DC maturation demonstrated by up-regulation of surface markers like MHC II and costimulatory molecules, only TLR signals induce cytokine secretion by DCs (33, 43). By comparing partially matured TNF-DCs, which do not produce cytokines, with fully matured LPS plus CD40-DCs in the Th1-mediated autoimmune model EAE, we have reported that only TNF-DCs suppress EAE by induction of MOG-specific CD4+ IL-10+ T cells (33). Here, we unravel the crucial role of concomitant activation of CD1d-restricted NKT cells for the development of this protective T cell response (Fig. 1). Soon after MOG/TNF-DC injections, we detected the release of Th2 cytokines by NKT and conventional CD4+ T cells (Figs. 2, 3, 5B, and 6B), indicating an early CD4+ Th2 state. The release of the Th2 cytokines is regulated by the CD1d-dependent NKT cell recognition of the endogenous ligand iGb3 (Fig. 7). In addition, both MHC II and CD1d molecules have to be expressed on the same DC (Fig. 6), suggesting simultaneous activation of the adaptive MHC II and the innate CD1d pathway by the injected DCs. For suppression of EAE by TNF-DCs, the secretion of IL-4 and IL-13 seems to create an intermediate Th2 deviation required for the subsequent differentiation of MOG-specific CD4+IL-10+ T cells (33), similar to what has been termed induction of Th2-like Tr1 cells in an asthma model (5, 44). Thus, DCs induce interdependently adaptive T cell and innate NKT network for efficient peptide-specific T cell responses. It further indicates how different DC maturation stimuli can decisively polarize the type of NKT cell response determining the subsequent CD4+ T cell differentiation.

The usage of Jn281−/− mice and reconstitution of these mice with invariant NKT cells demonstrated the involvement of invariant type I NKT cells for EAE prevention and induction of CD4+ IL-10+ T cells induced by MOG/TNF-DCs (Fig. 1B). However, EAE was more severe in CD1d−/− mice treated with TNF-DCs compared with Jn281−/− or to C57BL/6 injected with CD1d-deficient MOG/TNF-DCs (Fig. 1). This reduced EAE severity in Jn281−/− might be due to the presence of type II NKT cells, which are absent in CD1d−/− (45). It has been demonstrated that some of these type II NKT cells recognize myelin-derived sulfatides and participate in EAE prevention (30). Their activation following EAE induction might also contribute to the less severe EAE in mice treated with CD1d−/− MOG/TNF-DCs (Fig. 1C). Another possible factor for the reduced EAE severity observed with CD1d−/− MOG/TNF-DCs could be the activation of NKT cells by endogenous DCs. We and others have shown that injected DCs activate endogenous DCs resulting in altered immune responses (46, 47). Thus, type II NKT cells as well as endogenous DCs may account for the less severe EAE Jn281−/− and following treatment of WT mice with CD1d−/− DCs.

In this study, mice were injected with in vitro-generated and differentially stimulated DCs which had been pulsed with the MHC II-restricted MOG35–55 peptide but never with an exogenous ligand for CD1d. Blocking experiments using IB4, specifically recognizing the Galα1,3Gal linkage (42), indicated that NKT cells recognize, on both types of differentially matured DC, an endogenous ligand is presented on both types of and recognized by NKT cells (Fig. 7, A and B). This supports that NKT cell polarization rather relies on the costimulatory repertoire of the APC than on different CD1d ligands (28, 29). Furthermore, we show that the IB4 effect on serum cytokines is not due to altered DC maturation or CD1d-unspecific mechanisms (Fig. 7, B–D). Zhou et al. (31) have shown that IB4 blocks NKT cell recognition of plate-bound CD1d/iGb3 as well as exogenous and endogenous iGb3 presentation on DC, suggesting that iGb3 might be the CD1d ligand presented by the injected DC in our system. This is further supported by their finding that β-hexosaminidase B-deficient mice, lacking the enzyme to generate iGb3, exhibit a defect in NKT development and β-hexosaminidase B-deficient cells have a specific defect in generating lysosomal ligands 19 for NKT cells (31). However, so far iGb3 has not been identified in human or mouse tissues, therefore presentation of alternative glycolipids with terminal Galα1,3Gal might be possible.

For complete EAE protection induced by MOG/TNF-DCs, three repetitive injections are required, although a single injection of TNF-DCs already ameliorated the course of EAE (33). The tolerogenic capacity after repetitive injections of these partially matured TNF-DCs has also been reported in experimental autoimmune thyroiditis and collagen-induced arthritis (34, 35). This dependency on repetitive Ag administration for the induction of IL-10-producing Tr1 and prevention of autoimmune diseases has also been observed in the murine model of asthma (44). These Ag-specific Tr1 cells were termed Th2-like Tr1 because they produce IL-10 and IL-4 (5, 44). Also, gene expression analysis demonstrated a close relation between Tr1 and Th2 cells (48, 49). Thus, repetitive antigenic stimulations may promote the generation of CD4+IL-10+ regulatory T cells which could be due to incomplete differentiation. After MOG-specific in vitro restimulation of spleen cells, we observed unspecific IL-10 secretion when DC injections were combined with EAE induction (Figs. 4D, 5D, and 6C). The cellular source of this unspecific IL-10 is currently under investigation.

Using the exogenous CD1d ligand α-GalCer, it has been observed that single or repetitive injections activate NKT cells toward secretion of Th1 and Th2 cytokines or nonresponsiveness (25–27, 50). Thus, it is as yet unclear whether NKT cells can be selectively polarized by α-GalCer. However, multiple α-GalCer injections protect mice from MOG35–55-induced EAE (51, 52). As the mechanism of α-GalCer-induced EAE prevention, the induction of MOG-specific CD4+ T cells, producing IL-10 and IL-4, was identified (51). In mice treated three times with α-GalCer, they also measured decreased MOG-specific IFN-γ production, similar to what we observed by spleen cells from mice injected with MOG/TNF-DCs (Fig. 4C). This might indicate that MOG-specific CD4+ T cells from α-GalCer-treated mice are similar to the previously described IL-10-producing Tr1 cells (4, 5). Moreover, Kojo et al. (53) described the induction of regulatory DCs following three injections of α-GalCer able to suppress EAE by induction of IL-10-producing Tr1 cells. Our data are in agreement with those reports in which α-GalCer was used to activate CD1d-restricted NKT cells for preventing EAE by induction of CD4+IL-10+ T cells.
The recent reports that development of Ag-specific IL-10-producing Tr1 cells is linked to Th2 cells (48, 49, 54) supports our finding that NKT cells control the development of MOG-specific CD4+ IL-10+ T cells by regulating an early intermediate Th2 environment. Thus, the early transient CD1d-dependent Th2 environment following TNF-DC injections may favor the development of MOG-specific CD4+ IL-10+ T cells directly via IL-4R signaling (Fig. 4), suggesting a regulatory effect of NKT cells on CD4 T cells. Alternatively, the regulatory NKT cell effect might also rely on feedback signaling toward DCs, a mechanism described as “DC education” or “DC licensing” (40, 41). Several studies revealed the immunogenic adjuvant capacity of CD1d-restricted NKT for induction of CD4+ as well as CD8+ T cell responses (28, 55–57). Following in vivo loading of DCs with α-GaCer, NKT cell activation has been observed to induce DC maturation via CD40-CD40L signals, which results in their secretion of IL-12 and more efficient T cell stimulation (28, 55–57). Besides a direct effect of NKT cells on the MOG-specific T cells, feedback instruction of the injected DCs by NKT cells might also be possible.

DC feedback signaling is supported by our finding that both MHC II and CD1d have to be expressed on the same DC for the early DC-mediated Th2-biased cytokine environment followed by EAE suppression and development of MOG-specific IL-10+ T cells (Fig. 6). This pathway is further assisted by the recent report that multiple activations of NKT cells with MOG/TNF-DCs did not show EAE prevention and early release of EAE (33). C57BL/6 mice injected with MHC II-deficient MOG/TNF-DCs still prevent EAE (33). Nevertheless, this simultaneous and interdependent activation of innate CD1d-reactive NKT cells and adoptive MOG-specific T cells by the same DC might determine Ag specificity and reduce unspecific T cell activation. The precise mechanism regarding how CD1d-restricted NKT cells regulate this protective CD4+ T cell response is currently under investigation.

All studies using α-GaCer as adjuvant to enhance immunity by inducing DC maturation in vivo (28, 56, 57) or for treatment of EAE (21–23) left questions open regarding which type or types of APCs are mediating these polarizing effects on NKT cells. Our data suggest that semimature TNF-DCs can bridge T cells with innate CD1d-dependent NKT cells by the induction of type 2 cytokine secretion by T and NKT cells, which leads in our model to the induction of protective IL-10+CD4+ T cells. On the contrary, fully mature LPS plus CD40-DCs stimulate T and NKT cells to release type 1 cytokines which are not protective in the Th1-mediated autoimmune model EAE.

In conclusion, we provide evidence for a pivotal role of CD1d-restricted NKT cells in EAE prevention by MOG peptide-pulsed TNF-DCs. Induction of protective and nonprotective immune responses in EAE depends on the simultaneous and interdependent presentation of MHC II/self-peptide and CD1d/self-iGb3 to T and NKT cells by partially or fully matured DCs, respectively. Interaction between injected TNF-DCs, T, and NKT cells results in an early intermediate Th2 response which favors generation of IL-10+CD4+ T cells and EAE prevention.

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Disclosures

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